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Exhibit 17

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Non-additive effects of multiple amino acid substitutions on antigen-antibody recognition*

Synthetic peptides have been used to mimic the main antigenic site of foot-and-mouth disease virus (FMDV) of serotype C and of several variant isolates. This region includes multiple continuous B cell epitopes. The effect of single amino acid replacements, individually or in combination, on antigen specificity has been evaluated using monoclonal antibodies. Quantitative enzyme immunodot assays have shown that both additive and non-additive effects of multiple replacements occur in continuous B cell epitopes, with regard to antibody recognition. Antigenically critical single replacements may be compensated by other, non-critical replacements. Thus, the role of a single amino acid on antibody recognition depends on the sequence context in the antigenic domain. The non-additive effects of multiple replacements may modulate the extent of antigenic diversification of highly variable RNA viruses, and keep viruses confined within antigenic groups by precluding linear antigenic divergence.

1 Introduction

The effect of a single amino acid substitution on the structure and biological activity of a protein is often assumed to be independent of the effect of other replacements in the same protein. In this view, multiple replacements can be treated in a linearly additive fashion in structural and functional studies [1]. Accordingly, the effect of sequence changes on protein antigenicity is usually evaluated by replacing single amino acids within epitopes [2–4].

Very few attempts have been made to study the effect of multiple substitutions on immune recognition, as compared with the effect of each substitution individually. Previous evidence suggested that in continuous B cell epitopes multiple replacements resulted in a diminished antibody reactivity that was approximately equal to the product of the reactivities obtained with each of the replacements separately [5]. In contrast, Boyer et al. [6] have recently shown that the critical role exerted by a specific amino acid on the recognition of a T cell epitope depended on the presence of another amino acid two positions away in the same epitope.

Multiple amino acid replacements at defined domains are frequent among evolutionary variants of proteins, and particularly at the antigenic sites of highly variable RNA viruses [7–9]. Thus, it is important to investigate whether non-additive effects of amino acid substitutions occur in B cell epitopes, and what their relevance to antigenic diversification is.

We have identified B cell epitopes of foot-and-mouth disease virus (FMDV), an important animal pathogen that displays extreme genetic and antigenic heterogeneity [7–9]. The antigenic diversity of FMDV can be traced to variable regions of capsid proteins (VP) [8–10], in particular a disordered, flexible loop of VP1 that protrudes on the virion surface [11]. For FMDV of serotype C this site (termed site A, VP1 residues 138–150) is a cluster of multiple epitopes involved in virus neutralization [12–15]. Antigenic diversification at this site occurs both by accumulation of replacements with minor effects on antigenicity, and by single, critical substitutions that have a drastic effect on antibody reactivity [16]. Some of the replacements fixed during the natural evolution of the virus were introduced into substituted synthetic peptides that included site A. These peptides bound mAb to an extent similar to that quantitated with the corresponding variant VP1 or with the complete viruses ([14, 15, 17] and Mateu et al., unpublished results). In the present study, we have evaluated the effect on antibody reactivity of multiple substitutions at site A of FMDV type C that were fixed in the course of a disease episode [14, 18]. The results provide evidence both for additivity and for non-additivity of multiple substitutions on antigenic specificity.

2 Materials and methods

2.1 Antigens

The synthesis, coupling to KLH and quantitation of the peptides used have been described [17]. The peptides spanned VP1 amino acids 136–156 of FMDV type C, including antigenic site A (Table 1).

2.2 Antibodies

The mAb used were elicited against FMDV type C and recognize distinguishable, continuous epitopes that overlap in antigenic site A of FMDV C-S8 [12, 13, 15].

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Abbreviations: **EID:** Enzyme immunodot **FMDV:** Foot-and-mouth disease virus

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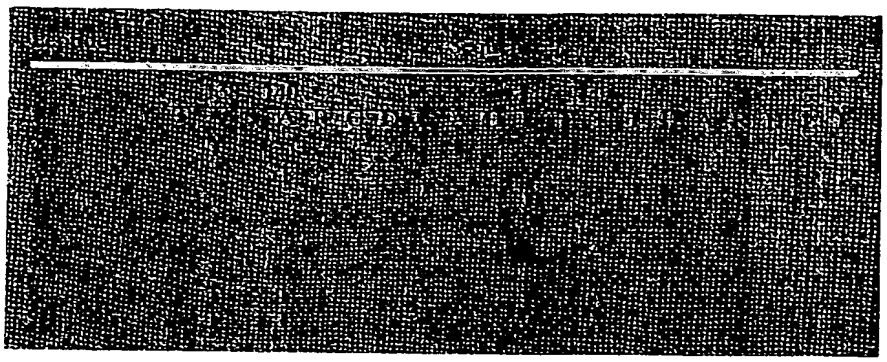
Table 1. Substituted peptides used in the present study



a) For peptides 2–9 only amino acids that differ from those of peptide 1 are indicated. The single letter code for amino acids is used. The horizontal line above the sequence delimits antigenic site A of FMDV C-S8 [15, 16]. Peptides 4 and 5 did not include the two N-terminal amino acids; this difference in length had no effect on the reactivity of any mAb, except for a slight decrease in binding of the shorter peptide to mAb SD6 and 5A2. This was taken into account when relative reactivities were calculated for peptides 4 and 5. Peptides 1, 8 and 9 represent sequences of FMDV isolates C-S8, C-S15 and C-S30, respectively, described in detail elsewhere [18].

2.3 Immunoassays

Quantitative enzyme immunodot (EID) assays were carried out essentially as described [14]. In brief, 10 pmol of each peptide were applied as a KLH conjugate to a nitrocellulose sheet using a dot-blot apparatus. After washing, the sheet was saturated with 3% BSA in PBS and incubated with dilutions of supernatant of hybridoma cultures. The sheet was then washed with 0.05% Tween 20 in PBS, as in all subsequent washing steps. This was followed by incubation with peroxidase-coupled goat anti-mouse IgG (2000-fold dilution) and thorough washing. The enzymatic reaction mixture was 0.015% H_2O_2, 0.5 mg/ml 4-chloro-1-naphtol, 20% methanol in PBS. The results were quantitated in a Chromoscan 3 (Joyce-Loebl, Gateshead GB) densitometer. With each antibody a relative reactivity was calculated for each peptide. This is defined as the ratio between the amount of product formed with the considered peptide and the amount formed with the reference peptide 1 (Table 1), expressed as a percentage.

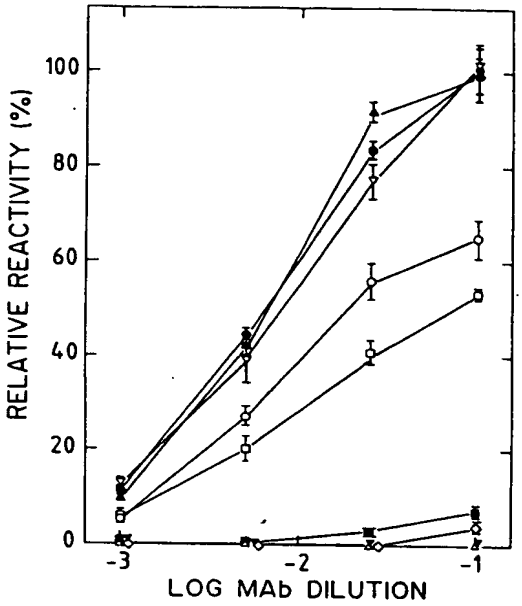


Figure 1. Reactivity in an EID assay of mAb 5A2 with substituted synthetic peptides representing FMDV sequences. The peptides tested are indicated in Table 1. Symbols ●, ○, ▲, △, ▼, ▽, ■, □, and ◇ are used for peptides 1 to 9, respectively. The relative reactivity of each peptide was calculated as described in Sect. 2.3; it is referred to the reactivity of peptide 1, with a 1 : 10 dilution of mAb. The experiment was carried out in quadruplicate, and SD are indicated.

3 Results

The relative reactivity of a set of substituted peptides that mimicked antigenic site A of FMDV type C was compared to that of a reference peptide in EID assays with a panel of mAb which recognize overlapping epitopes within this site [12, 13, 15]. The peptides tested included single or multiple replacements found in variant viruses from a single epizootics [14, 18] Table 1). As an example, the complete set of reactivities with mAb 5A2 is shown in Fig. 1. Reactivities with all nine available mAb, at one non-saturating concentration, are given in Fig. 2. The results with singly substituted peptides revealed that replacement $Thr^{149}\rightarrow$ Ala had no effect on mAb reactivity, and that substitutions $Ala^{138}\rightarrow$ Thr or $Ala^{140}\rightarrow$ Thr alone diminished the binding only of a few mAb (Fig. 2). Thus, these three substitutions could be considered noncritical regarding antigenic specificity. In contrast, replacements at position 147 had a substantial effect on the binding of most mAb. $Leu^{147}\rightarrow$ Ile greatly affected the binding of mAb SD6, 5A2 and, to a lesser extent, that of mAb 6D11. Substitution $Leu^{147}\rightarrow$ Val drastically diminished the binding of all mAb except 4G3 (Fig. 2). This result extends our previous observation with mAb SD6 [14], and those of others [4], against the view that chemically conservative substitutions in antigens do not affect antibody binding.

Then we examined the effects on antigenic specificity of multiple substitutions in one peptide. The relative reactivity expected from the additivity of the contributions of the individual replacements was compared with the relative reactivity actually observed with the corresponding multiple substituted peptide (Fig. 3). If additivity were operating, the observed value would equal the product of values

obtained with the singly substituted peptides [5]. This was in fact observed with the doubly substituted peptide 7 that includes Ala^{138}→ Thr and Leu^{147}→ Ile (compare Table 1 and Fig. 3 A). In contrast, no additivity of the effects of amino acid substitutions was observed with peptides 8 and 9, which included substitutions at positions 138, 140, 147 and 149, and which mimicked site A of variant viruses C-S15 and C-S30, respectively (Table 1 and Fig. 3 B and C).

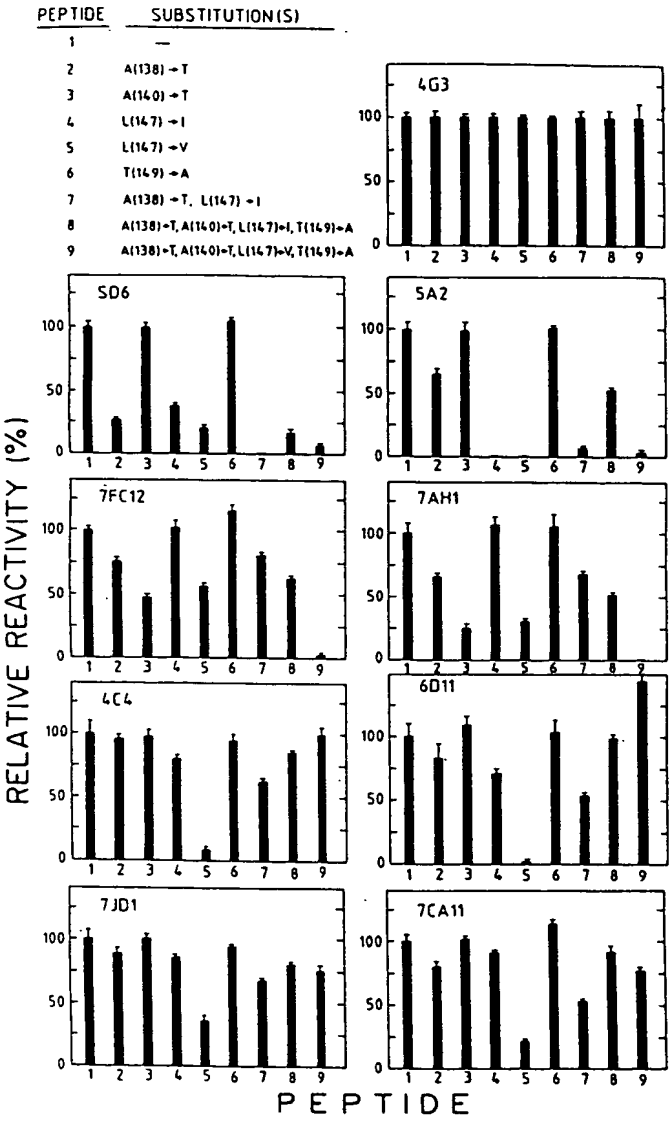


Figure 2. Reactivity in EID assays of mAb with substituted synthetic peptides. Each mAb (indicated at the top left of each panel) recognizes a different epitope on antigenic site A of FMDV C-S8 [12, 13, 15]. Several nonsaturating dilutions of each mAb were used in the experiments and similar results were obtained; only the results with one dilution of each mAb (1/5 to 1/200 of supernatant of hybridoma culture, depending on the mAb) are shown. The peptides used (Table 1) and the substitutions relative to the reference peptide 1 are listed in the upper left part of the figure. Each panel shows the relative reactivity (defined in Sect. 2.3) of each peptide in the set with the specified mAb. The assays were carried out in quadruplicate and in parallel, and standard deviations are indicated.

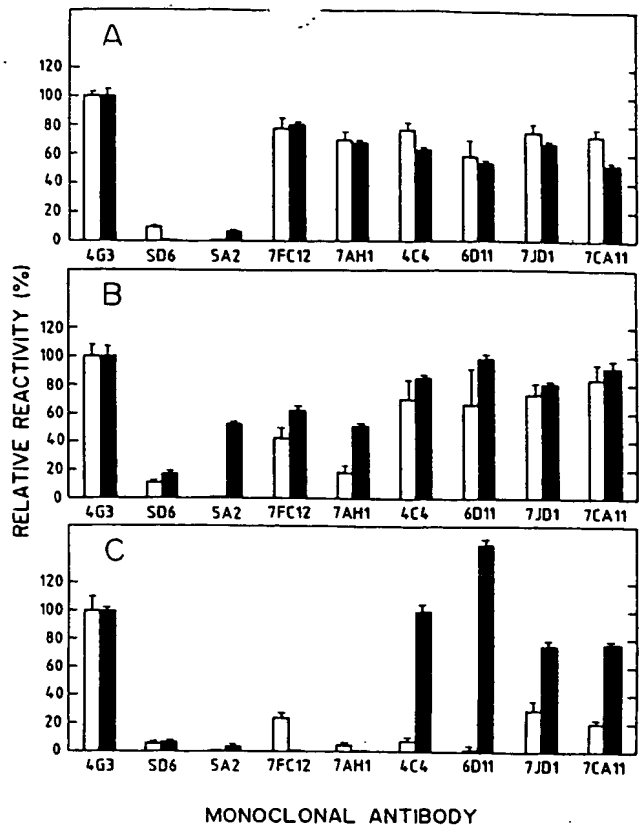


Figure 3. Comparison between expected and observed reactivities with mAbs of multiply substituted peptides. Panels A, B and C give the results with peptides 7, 8 and 9, respectively (Table 1). Relative to reference peptide 1, peptide 7 included two replacements (Ala^{138}→ Thr and Leu^{147}→ Ile), and peptides 8 and 9 incorporated the four replacements Ala^{138}→ Thr; Ala^{140}→ Thr; Leu^{147}→ Ile (peptide 8) or Val (peptide 9); Thr^{149}→ Ala (compare Table 1). Relative reactivities are defined in Sect. 2.3. For each multiply substituted peptide and for each antibody, the mean expected relative reactivity (empty bars) was calculated by multiplying the mean relative reactivities obtained with the corresponding singly substituted peptides (Fig. 2). The upper and lower limits of the expected reactivity were calculated by multiplying the upper and lower values (given by the standard deviations of Fig. 2), respectively, of each individual reactivity of the singly substituted peptides. A similar range was obtained when the expected reactivity was calculated for each of the four duplicates of the experiment, and then the SD of the four values was determined. The observed relative reactivities (filled bars) were obtained from EID assays with the multiply substituted peptides, as given in Fig. 2. The mean values and SD of four duplicates carried out in parallel are presented. An example of such calculations for the multiple substituted peptide 7 and mAb 7CA11 follows: The mean experimental relative reactivities and standard deviations obtained with the corresponding singly substituted peptides 2, 3, 4 and 6 were 80 ± 4%, 101 ± 3.5%, 91 ± 2% and 115 ± 3% (Fig. 2, mAb 7CA11). The expected relative reactivity for peptide 7 was (80/100) × (101/100) × (91/100) × (115/100) = 85%; the upper value of the expected reactivity, given by the standard deviations was [(80 + 4)/100] × [(101 + 3.5)/100] × [(91 + 2)/100] ×[(115 + 3)/100] = 96% and the lower value was [(80 − 4)/100] × [(101 − 3.5)/100] × [(91 − 2)/100] ×[(115 − 3)/100] = 74%. Thus, the expected relative reactivity is 85 ± 11% to be compared with the experimental relative reactivity of 92 ± 5% (panel B, mAb 7CA11). The calculations were also carried out from data obtained with a fivefold lower concentration of each mAb, and equivalent results were obtained (not shown).

The non-additive effects of multiple substitutions in peptide 8 were clearly revealed with mAb 5A2, and to a lesser degree with 7AH1 (Fig. 3B). With peptide 9, non-additivity was observed with mAb 7FC12, 7JD1, 7JD1, 7CA11 and most clearly with 4C4 and 6D11 (Fig. 3C). Due to the critical effect of the single substitution $Leu^{147} \rightarrow Val$ (Fig. 2), minimal binding was expected to peptide 9, that included this same replacement; the expected relative reactivities were about 8% and 2% for mAb 4C4 and 6D11, respectively. However, no diminished binding of these two mAb to peptide 9, relative to peptide 1, was observed (Fig. 3C). A similar though less pronounced discrepancy applies to mAb 7JD1 and 7CA11 (Fig. 3C). In agreement with these results, mAb 4C4, 6D11, 7JD1 and 7CA11 reacted in Western blot assays with VP1 of FMDV C-S30 to a similar extent as with VP1 of C-S8, mimicking the results with the homologous peptide 9 (data not shown). We conclude that both additive and non-additive effects of multiple substitutions on antigenicity were revealed with mAb directed to a major antigenic site of FMDV.

4 Discussion

In the evaluation of our approach to using synthetic peptides and mAb to evaluate antigenic specificity, several considerations are relevant. Synthesis of peptides by classic solid-phase procedures and purities >95% were required for reliable quantitations of differences in reactivity. Due to the limited purity of the products, multiple pin peptide synthesis [2] was not adequate for our purposes. Even at the highest coupling efficiencies [19], this procedure would yield typically <60% purity for peptides of 12 to 15 residues, the minimum length required to include most of the epitopes identified in antigenic site A [12–15].

It could be argued that the results with mAb, needed for understanding the physical basis of antigen-antibody recognition, may not be relevant to the polyclonal response *in vivo*. However, sera from animals immunized with FMDV C-S8 (site A sequence represented by peptide 1, Table 1) recognized the substituted peptides with relative reactivities comparable to the average obtained with the mAb used (data not shown). We are currently extending these analyses to quantify the reactivity of sera from FMD-convalescent cattle and swine with the modified peptide antigens.

Early studies of serological cross-reactivity with evolutionary variants of some proteins showed a good correlation between immunological distance and the total number of amino acid differences between them [20 and references therein]. However, our results and those of Boyer et al. [6] have revealed that in some cases accumulation of replacements can influence antigenicity to an extent that does not result from the mere linear addition of the effects of each single replacement alone.

In the FMDV epitopes studied, VP1 residue 147, substituted in five of the peptides analyzed (Table 1), is preceded by two residues, Ala^{145} and His^{146}, that were identified as critical for site A antigenicity, based on the behavior of virus mutants [15, 16]. The results described here suggest that critical replacements at the central, relatively conserved core of site A (residues 141 to 147 in Table 1; see [8, 9, 14–16] can occasionally be compensated by further, non-critical replacement with substantial recovery of antigenic specificity. This was the case with substitution $Leu^{147} \rightarrow Val$, when accompanied by other replacements in FMDV C-S30 (Table 1 and Figs. 2 and 3C). The compensatory effects of this type may modulate the extent of antigenic diversification of hypervariable domains of rapidly evolving viruses such as FMDV or HIV-1 [8, 9, 21, 22]. Such effects may contribute to maintaining the identity of FMDV within defined serotypes by precluding continuous, linear diversification.

Synthetic peptides are promising candidate vaccines [23, 24], as evidenced also for FMDV [25, 26]. However, antigenic variation may pose a serious obstacle to the use of chemically defined vaccines. In our view, it is important to recognize the molecular basis of the different antigenic specificities of co-circulating viruses [16, 17], and the effect of single and multiple substitutions on such specificity. This information should then be used to choose appropriate peptide mixtures for vaccine formulation. The lack of three-dimensional structural data for FMDV of serotype C (or for the antigenic domain A) prevents interpretation at the molecular level of the effects that the amino acid replacements described in this paper have on the conformation of site A.

The results reported here and those of Boyer et al. [6] point to the unpredicted coordinated effects of multiple amino acid substitutions in B cell and T cell epitopes on the antigenic specificity of evolutionary variants of proteins. Thus, the concept of antigenically "critical" residues should not be considered an absolute term, but a relative one that depends on the amino acid sequence context.

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